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Degenerative processes in the marginal retina of the tench (*Tinca tinca*)

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SUMMARY

By cryolesion of the scleral-corneal perimeter of the eye of a teleost we eliminated both the marginal growth ring and the immature portions adjacent to the neural retina. Electron microscopy revealed necrotic destruction of the whole of the germinative zone and a large number of apoptotic cells in neighbouring zones. In the degenerative processes that follow the lesion, at least three types of cells are involved: microglial cells, pigmentary cells and extravascular heterophilic granulocytes.

Both microglia and the pigmentary cells act as phagocytes and participate in the elimination of debris from the lesioned area. Heterophilic granulocytes probably collaborate in the modification of the environment for the subsequent regeneration. During the degenerative processes after the lesion, all three cell types participate in the transformation of the damaged retinal zone into a favourable medium for later regeneration of the neural tissue.

Key Words: Apoptosis - microglia - necrosis - teleosts - ultrastructure.

INTRODUCTION

The retina of the teleosts grows throughout the life of the animal thanks to the continual addition of cells. These originate both from a population of disperse neural progenitors in the mature retina, known as rod precursors, which only originate photoreceptors of the rod type in control animals (Lyall, 1957, Sandy and Blaxter, 1980; Johns and Fernald, 1981), and, above all, from the circumferential germinal zone (CGZ) (Boucher and Hitchcock, 1998), which is located on the peripheral margin of the retina (review in Fernald, 1991). Furthermore, the retina of the teleosts constitutes an interesting model for the study of the response to lesions of the central nervous system (CNS) because of its capacity to regenerate, both morphologically and functionally, damaged retinal zones (reviews in Hitchcock and Raymond, 1992; Raymond and Hitchcock, 1997).

Studies of the processes occurring in the fish retina after mechanical (Hitchcock et al., 1992; Cameron and Easter, 1995) or chemical lesions (Maier and Wolburg, 1979; Raymond et al., 1988; Negishi et al., 1991), which address the neuronal response during regeneration, are relatively frequent. By contrast, the degenerative processes that appear after retinal lesion to these animals have received little attention (Maier and Wolburg, 1979; Jimeno et al., 1999).

It is known that after the production of a lesion of the CNS, the tissue remnants originated are phagocytosed by diverse types of cells, among which microglial cells and some types of blood cells are outstanding. Previous studies have demonstrated the functions of the microglial cells in the visual pathway of fish after a traumatic lesion: the elimination of remains (Springer and Wilson, 1989; Dowding et al., 1991; Battisti et al., 1995; Velasco et al., 1995) and, as in other models, the release of factors that affect the adjacent area (Giulian et al., 1986; Leibovich et al., 1987). Nonetheless, there are few data on the role of these cells in the retina of fish (Dowding et al., 1991; Velasco et al, 1995; Jimeno et al., 1999).

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Other cells that develop phagocytic capacity in the retina of vertebrates as an indispensable condition for its correct functioning are the cells of the retinal pigment epithelium (RPE). Under normal conditions, these cells participate in the renovation of the outer segments (OS) of photoreceptors (PR) (for a review, see Steinberg, 1985). However, their participation as phagocytic elements in degenerative processes originated by lesions in the retina of fish has not been described.

Here we report an electron microscopy (EM) study of the cellular and tissue modifications of the retina of a species of teleost fish during the degenerative processes originated by elimination, using a cryolesion technique, of its marginal proliferative zone.

Materials and Methods

All procedures used in this work were in accordance with the guidelines of the European Communities Council Directive (86/609/EEC) and current Spanish legislation for the use and care of animals.

For this study 27 adult tench (*Tinca tinca* L., 1758, Cyprinidae, Teleostei) were used. They were obtained from a commercial hatchery and kept in aquaria at 18-20°C. The animals were anaesthetised with 0.03% tricaine methanesulphonate (MS-222, Sigma) and the sclero-corneal zone of the right eye was lesioned with a cryogenic system (Cry-AC, Brymill) for 5s.

The tench were kept in aquaria until sacrifice at 2, 15, 30, 60, 90, 120, 180 or 240 days post-injury. The animals were anaesthetised again, and perfused intracardially with 50 ml of isotonic saline solution (0.63% NaCl in distilled water) containing 5 UI/ml heparin and fixed with 150-200 ml of a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde and 0.05% CaCl2 in 0.1 M cacodylate buffer (pH 7.4). The eyes were removed, the lenses extracted, and the retinas cut into pieces and stored in the same solution overnight at 4°C.

The pieces were washed in 0.1 M cacodylate buffer (pH 7.4) with 0.18 M sucrose and 2 mM CaCl2. Post-fixation was carried out in 1% OsO4 in distilled water containing 1% potassium ferricyanide for 2 h. Before dehydration, the pieces were subjected to block staining with 1% uranyl acetate in distilled water. Dehydration was performed using a graded series of cold ethanol and Epon 812 (Fluka) was employed as embedding resin.

Semi-thin sections were stained with toluidine blue and ultra-thin sections were mounted on Formvar-coated one-hole grids, contrasted with uranyl acetate and lead citrate, and studied using a ZEISS EM-900 electron microscope.

Results

An increase in the population of microglial cells caused by cryolesion of the circumferential growth zone (CGZ) has been described in the whole of the retina, (Jimeno et al., 1999), although this is the only known modification of the mature retina as a result of elimination of the marginal ring. Our results demonstrate that, in this model, the degenerative reactions are limited to the lesioned zone itself and to the adjacent zones; i.e., to the germinative and maturing portions of the peripheral retina. Therefore, in this study, our descriptions of degenerative processes focus on these zones of the retina of adult teleosts.

The CGZ of the retina of control animals was a narrow ring formed by a dense packing of undifferentiated cells (Fig. 1A). Between the CGZ and the mature retina there was a zone of differentiation a few micra wide (Fig. 1A), in which vitreal and scleral portions could be distinguished. The vitreal zone was composed of inner plexiform and ganglion cell layers, both well differentiated, as well as a very thin layer of the axons of the ganglion cells. The scleral portion was less well defined and consisted of a dense accumulation of cells in diverse states of differentiation, which on maturing originated the photoreceptor layer, the inner and outer nuclear layers and, between these later, the external plexiform layer.

In animals sacrificed two days after the lesion the CGZ was completely destroyed. What was the marginal ring was occupied by necrotic tissue, with no survivors from among the cells that formed this zone at the time of injury. The cellular debris showed the typical characteristics of traumatic death, such as swelling and rupture of the nuclear envelope. In the zone of differentiation, adjacent to the CGZ, most of the cells were in different states of apoptosis and had the morphological characteristics typical of this process: cell shrinkage and characteristic condensations of chromatin in nuclei with an intact envelope (Fig. 1B,C).

Both among the debris of the CGZ and in the adjacent zone of differentiation there were numerous very active phagocytes with very euchromatic nuclei and an organelle-rich cytoplasm (Fig. 1D).Most of these cells were identifiable as microglia and their cytoplasm contained phagosomes with cell remains and even complete cells in an advanced state of apoptotic degeneration.

In this post-injury period, granular (heterophils) leukocytes were very frequently found in the zone of lesion. These heterophils featured a large number of cytoplasmic granules with an axial electron-dense crystalloid (Fig. 1E). The localisation of these cells in this post-lesion period indicated that they were from the RPE (Fig. 1B,E).

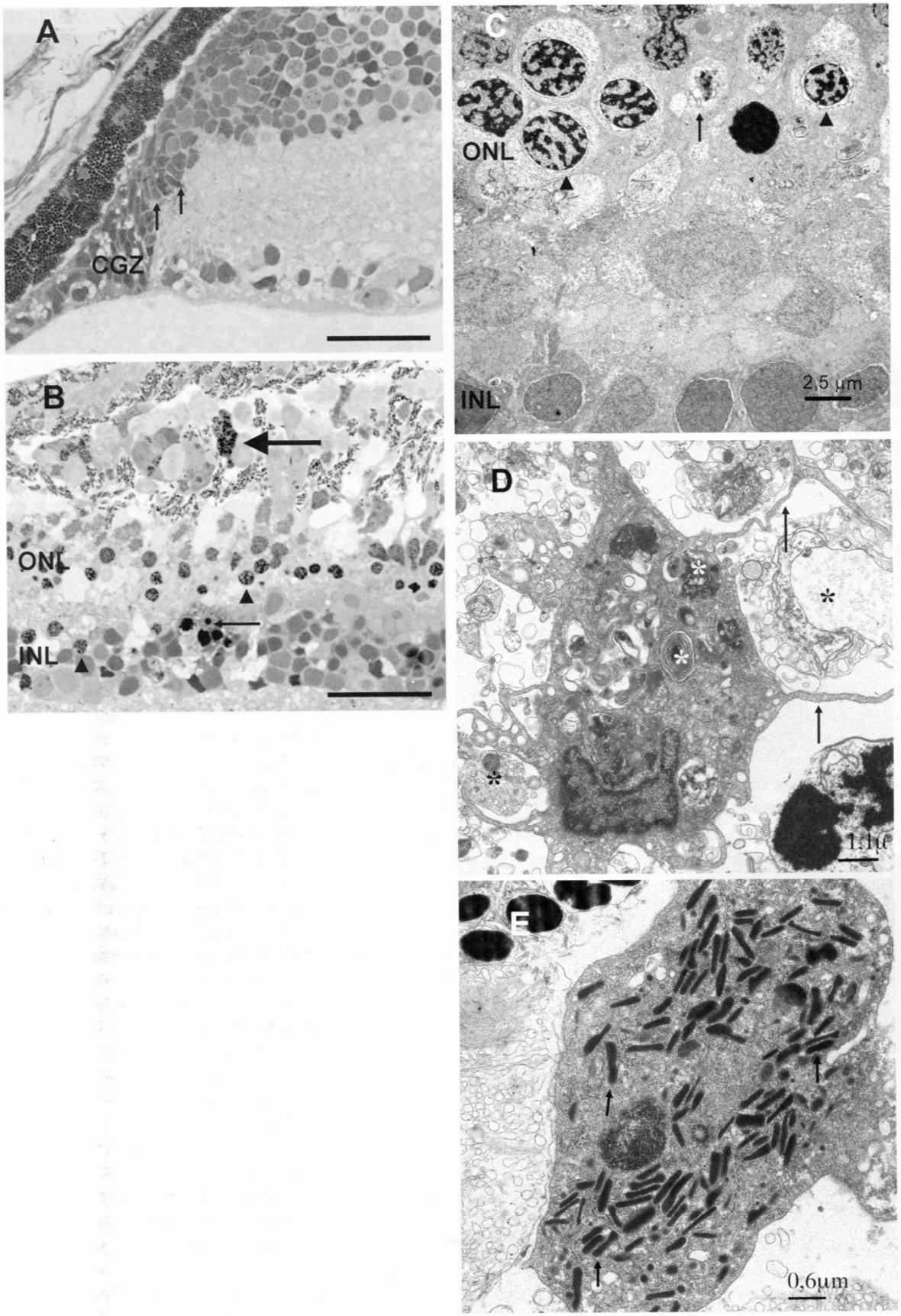

Fig. 1.– A and **B:** Semi-thin sections (1 µm thick) stained with toluidine blue. Scale bar: 50 µm **A:** Circumferential growing zone (CGZ) from a control retina showing proliferative cells (arrows). **B:** Adjacent portion of the CGZ 2 days after lesion. Numerous necrotic (small arrows) and apoptotic (arrowheads) cells in the ONL and INL can be seen. Note a granulocytic cell (big arrow) penetrating among the photoreceptor's segments. **C,D** and **E:** ultra-thin sections from a 2 days post-lesioned retina. **C:** Necrotic (arrow) and apoptotic (arrowheads) cells in the ONL of the portion adjacent to the lesioned CGZ. **D:** Microglial cell in the lesioned CGZ forming cytoplasmic expansions (arrows) to remove the cell debris (black asterisks). The phagocytic cell has a very euchromatic nucleus and many phagosomes in its cytoplasm (white asterisks). **E:** Granulocytic cell (heterophil) embedded among the RPE cells and the photoreceptor's segments showing typical ovoid granules (arrows).

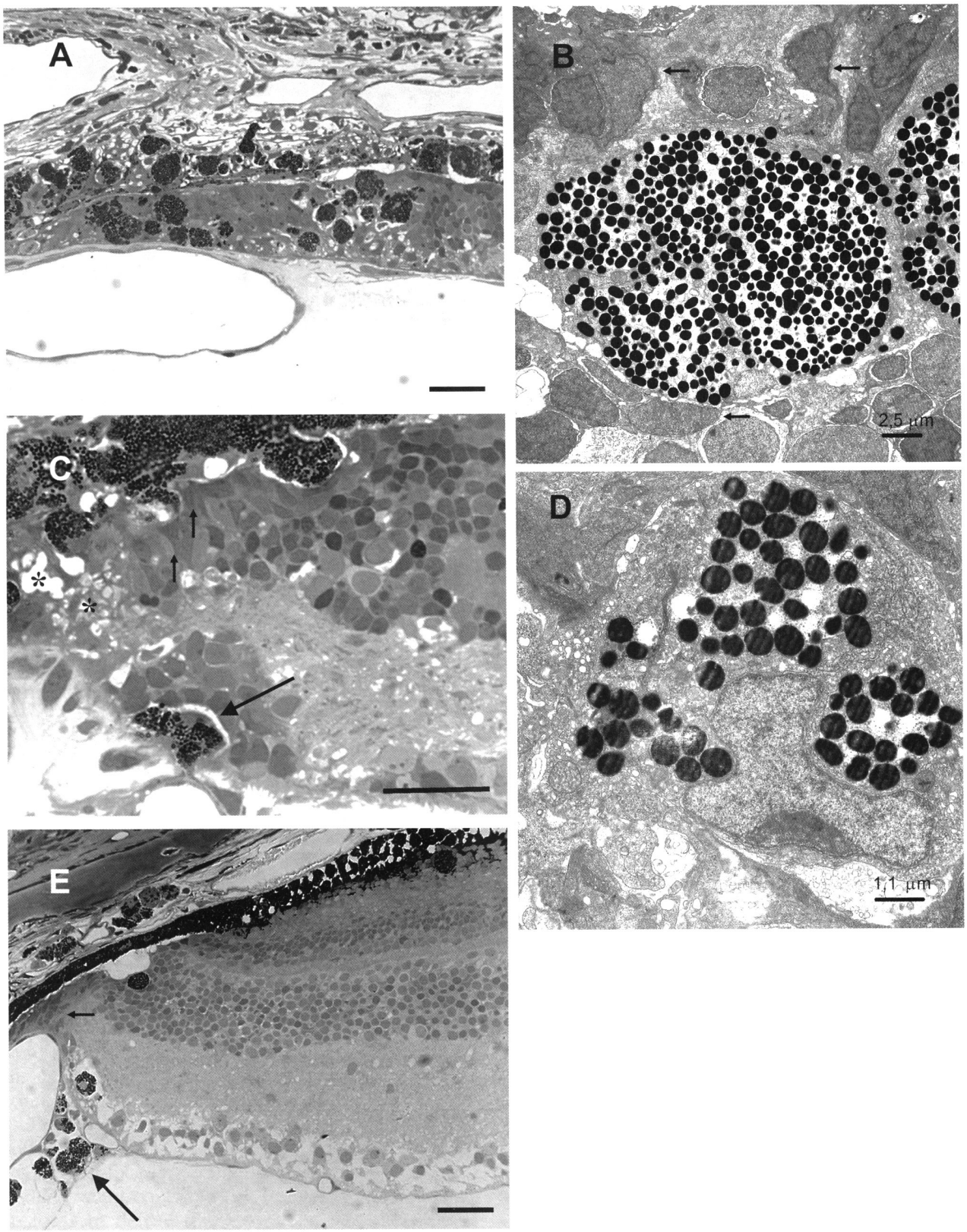

Fig. 2.– A, C and **E:** Semi-thin sections (1 µm thick) stained with toluidine blue. Scale bar: 50 µm **B** and **D:** Ultra-thin sections. **A:** Post-lesion retina at 15 days. The lesioned portion is occupied by round cells containing many pigment granules. **B:** Magnification of a pigmented cell present in the neural retina. Arrows: proliferative cells. **C:** Post-lesion retina at 30 days. The proliferative cells (small arrows) have still not reconstituted the CGZ. In the area where the CGZ had formerly been, some holes due to the necrosis can be observed (asterisk). Large arrows: cells filled with pigment in the neural retina. **D:** Pigmented cells in the vitreal-most part of the retina at 30 days post-lesion. **E:** Peripheral retina 240 days post-lesion. The CGZ is regenerated (small arrow) and still there are some pigmented cells embedded in the neural retina. Some of these cells (large arrows) are in the connective tissue.

Fifteen days after the cryolesion, the population of necrotic cells had diminished in an evident way. Nevertheless, apoptotic cells continued to be very abundant, particularly in the presumptive ONL (Fig. 2A). In this period, phagocytes became less abundant in the lesioned zone, while most of the heterophils infiltrated in the neural retina were located in scleral portions, in particular close to the outer segments of the photoreceptors.

In the damaged retina of all the cryolesioned animals sacrificed between 15 and 240 days after the injury the presence of numerous RPE cells was very notable. These cells were mainly located in the lesioned zone and its neighbourhoord (differentiation zone), although some were located in any of the layers of the mature retina, at a distance from the damaged marginal ring. The RPE cells infiltrated in the neural retina became round in shape and their cytoplasm contained both pigment granules and myeloid phagosomes (Fig. 2B).

Between 30 and 240 days post-lesion apoptotic cells were scattered throughout the presumptive ONL of the maturation zone. As the post-lesion period progressed the apoptotic cells became scarcer.

In animals sacrificed after post-lesion periods of more than a month, microglial cells with abundant phagosomes could be visualized in the vitreal portions of the retina.

The RPE cells infiltrated in the neural retina, which during the first month post-lesion were distributed throughout the scleral-vitreal extent of the retina, were mainly located in vitreal portions in longer post-lesion periods (Fig. 2C, D, E) In the longest post-lesion periods of those analysed (240 days), after crossing the whole of the neural retina some of these RPE cells reached zones of connective tissue associated with the vitreous humour (Fig. 2E).

Discussion

The retina of some fish (reviews in: Hitchcock and Raymond, 1992, Raymond and Hitchcock, 1997) and amphibians (Stone, 1950, Sologub, 1968) are capable of morphological and functional regeneration after diverse types of lesions. Studies on the regenerative processes in these models are abundant. By contrast, however, the processes occurring prior to the beginning of regeneration have not been studied in depth.

Although these processes are usually referred to as "degenerative", they should be differentiated from the degeneration originated by lesions to the retina of mammals. Whereas in mammals this process concludes with the elimination of the damaged tissue and the formation of a scar, in fish and amphibians the lesioned zone must be prepared for later effective regeneration at the same time as the damaged tissue is eliminated.

In a previous work, we conducted a light microscopy study of the modifications of the microglia taking place during degeneration in this experimental model of retinal lesion using morphological and histochemical techniques (Jimeno et al., 1999). Nonetheless, in the degenerative processes in the model used here, as well as the microglia, other types of cells participate that are not identifiable by light microscopy and which are of importance in the creation of propitious conditions for posterior regeneration.

In the tench retina, under normal conditions the microglia constitute a scant population of small polymorphic cells (the resident microglial; Jimeno et al., 1999) which is the equivalent of the highly ramified resident microglial in mammals (Rio Hortega, 1920; Streit, 1995). Furthermore, the existence of ameboid microglia in the retinas of adult tench is noteworthy (Jimeno et al., 1999). In mammals this type of microglia is only present during development. In teleosts, its presence in the retina, optic nerve and tectum of adult animals is associated with the continuous growth of the visual system of these animals (Velasco et al., 1995).

The results of this work concerning the morphological changes of the microglia and their temporal sequence are consistent with those reported using histochemical methods (Jimeno et al., 1999). Tomato lectin and NDPase reveal increases in the population of cells that react to these markers and electron microscopy discloses that these cells have the ultrastructural characteristics of microglial cells. The morphology of these cells in the lesioned zone is ramified and with the typical characteristics of very active phagocytes.

As well as the histochemically-labelled microglial cells uncovered using light microscopy, electron microscopy allows the identification of other cell types that participate in the degenerative processes of the lesioned retina, such as cells from the RPE and blood cells.

In the retina of control animals the main function of RPE cells is phagocytosis of the shed portions of the outer segments of photoreceptors (for a review, see Steinberg, 1985). There are no data available about any activity of these cells in relation to the degenerative processes originated by lesions. Nevertheless, among the results of the present work the location of RPE cells in all layers of the lesioned retina is noteworthy. We did not find significant ultrastructural differences between these cells and the RPE cells of unlesioned animals, although when they are present in the lesioned neural retina their shape is rounder than when they are in their original location in the pigment layer. It cannot be ruled out that some of these cells might be

macrophages which have phagocytosed granules of pigment from the cytoplasm of RPE cells damaged by the cryolesion.

In the retina of amphibians RPE cells participate actively in the regenerative processes that follow a lesion. These cells are able to transdifferentiate and regenerate the neural retina of these animals (Stone, 1950; Keefe, 1973; Okada, 1980; Klein et al., 1990). By contrast, the regeneration of the neural retina of fish by transdifferentiation of cells from their pigmentary epithelium is not considered possible (Knight and Raymond, 1995).

We are in agreement with the above authors because we never observed any mitotic figures among the RPE cells and because in the neural retina all these cells contained melanin granules in their cytoplasm, and the loss of these granules is one of the first steps required in the transdifferentiation process (Stroeva and Mitashov, 1983; Reh and Levine, 1998). We believe that RPE cells from the surroundings of lesion could act, in some way, as migrating phagocytes inside the lesion, helping the microglial cells to remove all the cellular debris.

Some "wandering" phagocytes in the retinas of fish have been described, mostly among the RPE cells (Braekevelt, 1980). However, the cells that we observed in this layer of the tench retina could be clearly identified as heterophils owing to the characteristic granules with an axial crystalloid that filled the cytoplasm. It is known that in some pathologic dysfunctions, these cells can behave as phagocytes (Hine, 1992), but this behaviour was never observed in our model. In the scleral-most parts, these cells were seen to be filled with their typical granules, although in the inner and vitreal parts these granules were fewer in number. This suggests that during the degenerative process these cells release substances from the granules that are able to modify the medium (Galli et al., 1984; Vallejo and Ellis, 1989), favouring the later regenerative processes.

In this work we used an effective method –the cryolesion– to eliminate the proliferative growth zone and the adjacent immature retina. At EM level we found that the proliferative cells of these two portions had been destroyed and all of them were necrotic or apoptotic, depending on their location. Furthermore, we observed that at least three kinds of cells showed a reaction to this lesion: microglial cells, RPE cells and some types of blood cells. Both microglial and RPE cells act as phagocytes, removing all the cell debris from the area of the lesion, and the blood cells could release the substances contained in their granules to the environment.

We know that after this kind of retinal damage the CGZ recovers its original state (Jimeno et al., 1999) and therefore we propose that the role of all these cells at the site of the lesion after cryolesion is very important for later regeneration of this retinal portion, probably because one of their functions is to transform the degenerative area in a manner favouring the later regeneration of the neural tissue.

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